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Cellular Proteins Bind to the 3′ End of Sindbis Virus Minus-Strand RNA

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Forty-four nucleotides at the 5′ terminus of the genomic RNA of Sindbis virus can form a stable stem-loop structure and have been shown previously to be important for viral replication. The structure formed by the complement of this sequence at the 3′ end of the minus-strand RNA has been proposed to be a promoter for RNA replication and as such might be bound in a specific fashion by proteins of either cellular or viral origin. Short oligonucleotide probes (either 62 or 132 nucleotides) representing the 3′-terminal sequence of the minus strand were prepared. When added to extracts from infected or uninfected cells, these probes were bound by cellular proteins, as evidenced by a shift in the electrophoretic mobility of the (labeled) oligonucleotide. Competition experiments confirmed the specificity of the interaction. Proteins of apparent molecular sizes 42 and 44 kDa, and to a lesser extent 52 kDa, could be cross-linked to the minus-sense probes by UV irradiation. A mutant minus-strand probe identical to the longer probe except for a single-nucleotide deletion corresponding to nucleotide 5 in the genomic RNA, which is lethal for the virus, was also found to bind the same proteins as the wild-type probe. The half-life of the mutant probe-cellular protein complex was threefold longer than that of the wild-type complex, however, indicating that the mutant probe was bound more tightly than the wild-type probe. We hypothesize that the binding of cellular factors may be transiently required for initiation of transcription of plus-strand RNA from the minus-strand template and that overly tight binding of such factors is deleterious for RNA replication.

The RNA replicases of RNA animal viruses are poorly understood. In the case of the alphaviruses, which are enveloped plus-strand RNA viruses, four nonstructural proteins, termed nsP1 to nsP4, are encoded by the virus and all four have been shown to be essential for RNA replication (12, 13). Currently it is believed that nsP1 is required for capping of viral RNAs (23) and for initiation of minus-strand RNA synthesis (13), that nsP2 is both an RNA helicase required for RNA replication (11) and a proteinase required for processing the nonstructural proteins from a polyprotein precursor (14), and that nsP4 is the viral RNA polymerase (3, 12). Alphavirus replicases have been partially purified from infected cells, and the major virally encoded component has been found to be nsP1, with minor amounts of nsP2 and nsP4 (see reference 35 for a review). However, the replicases are not capable of specific initiation of RNA synthesis and have proven recalcitrant to further purification.

In addition to the virally encoded components, there are reasons to believe that cellular components might form part of the alphavirus replicase, as has been found in the case of other RNA viruses. The RNA replicase of phage Qβ contains three host cell proteins and one viral component (5), while the purified polymerase complex of cucumber mosaic virus contains two virus-encoded proteins and one host factor (15). Although the alphavirus replicase has not been purified sufficiently to identify host proteins that might be specifically associated with the replicase, the different effects of mutations in different cell lines have led to the hypothesis that host cell factors bind to conserved nucleotide sequence elements in the RNA to promote RNA replication (18, 25, 26). In the current model, these cellular proteins, probably in association with viral replicase components, recognize an RNA sequence or structure and interact with it in order to catalyze initiation of RNA synthesis. The participation of host proteins in the recognition of these viral sequence elements would require that these sequence elements be conserved during evolution of the virus.

Four conserved domains in the genomes of alphaviruses have been proposed as recognition sequences for the replicase complex (27–29). The best-characterized sequence element is a 24-nucleotide promoter containing the start site of the subgenomic RNA which has been shown to be necessary and sufficient for transcription of a subgenomic RNA (21, 32). The importance of three other elements, a conserved 19-nucleotide sequence adjacent to the poly(A) tail, a 51-nucleotide element capable of forming two hairpin structures found in the coding region of nsP1, and the 5′-terminal 44 nucleotides, have been confirmed by mutational analyses (18, 25, 26).

The mutational analysis of the 5′ nontranslated region (NTR) of Sindbis virus RNA (25) demonstrated that deletions within the first 44 nucleotides were deleterious for virus growth. Deletion of the first residue (A), of residues U2-U3-G4, or of residue A5 was lethal. Deletion of residue G8 or of residue C36, which are hypothetically base paired in the stem-loop structure, led to thermosensitivity of the rescued viruses. All other deletions examined within the first 44 nucleotides gave rise to virus that was severely impaired for growth in chicken cells or in mosquito cells, or in both. These results suggested that the stem-loop structure that can be formed by these 44 nucleotides, whether in the plus strand or in the minus strand, was important for the replication of Sindbis virus RNA. Furthermore, since the effect of any particular mutation often differed dramatically in chicken cells and in mosquito cells, both of which represent natural hosts of the virus, cellular proteins might bind to this structure.

In this study, we examined the ability of the 3′-terminal

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region of Sindbis virus RNA minus strand, and of mutagenized derivatives, to interact with proteins from chicken cell extracts. We found that labeled probes from this region were bound by chicken proteins in a specific fashion and that a mutant probe was bound even more tightly by these same proteins. The results are consistent with the hypothesis that these cellular proteins are involved in initiation of plus-strand RNA from a minus-strand template.

MATERIALS AND METHODS

Preparation of RNA transcripts. The wild-type Sindbis virus in these studies was derived from a full-length cDNA clone of Sindbis virus, pToto51 (18), by transfection of chicken cells with RNA synthesized *in vitro* with SP6 RNA polymerase as previously described (33). p5NTd(5) is a mutagenized derivative that has nucleotide A5 deleted, which is lethal so that no virus can be rescued from this clone (25). Viruses derived from two other mutagenized derivatives, p5NTd(8), which has nucleotide G8 deleted, and p5NTd(8,36), in which both G8 and C36 are deleted, are thermosensitive (25). Wild-type Sindbis virus RNA or RNAs from Sindbis virus mutants 5NTd(8) and 5NTd(8,36) were used as templates for cDNA synthesis, using a primer annealing to nucleotides 126 to 145. This same primer was also used to prime first-strand DNA synthesis from the full-length cDNA clone p5NTd(5). The first-strand DNA products were amplified by the polymerase chain reaction using a primer containing an *Eco*RI restriction site followed by the first 11 bases of either Sindbis virus 5NTd(8), 5NTd(8,36), or 5NTd(5), respectively, together with the original cDNA primer. The products were digested with *Hin*dIII and *Eco*RI, purified by gel electrophoresis, and inserted into vector pGemini 4 (Promega) prepared by digestion with the same enzymes (pGEH series of clones). A shorter cDNA representing the 62 first bases of Sindbis virus RNA was obtained in the same way, using an oligonucleotide primer annealing to nucleotides 46 to 67 but containing a mismatch at position 58 (C instead of G) and a T inserted between nucleotides 57 and 58, so that a unique *Xba*I restriction site was introduced at positions 54 to 59 (pGEX).

A derivative of pBluescript II (Stratagene) in which the *Bss*HII restriction fragment had been replaced by an oligonucleotide carrying the sequence of SP6 and T7 RNA polymerase promoters in opposite orientations, separated by *Eco*RI and *Hin*dIII restriction sites, was constructed as a control plasmid (pBEH).

Plasmids pGEH (or pGEX), pGEHd(5), pGEHd(8), and pGEHd(8,36), containing the wild-type Sindbis sequence or the corresponding mutagenized sequences, were transcribed with T7 RNA polymerase after linearization at the unique *Eco*RI restriction site, to give minus-sense probes. A plus-sense probe was also transcribed from plasmid pGEH with SP6 RNA polymerase after linearization at the unique *Hin*dIII restriction site. pBEH was transcribed with SP6 polymerase after linearization at the *Pvu*II site to produce a probe of approximately the same length as that from the pGEH series for use as a control. Transcription reactions were performed at 38°C for 15 min in the presence of 10 μCi of [α-^{32}P]UTP and -CTP (800 Ci/mmol; Amersham), 500 μM ATP and GTP, 100 μM UTP and CTP, and either SP6 or T7 RNA polymerase. These ^{32}P-labeled transcripts were used as probes in binding assays. Alternatively, the transcripts were labeled with 10 μCi of [^{3}H]UTP (Amersham) in the presence of 500 μM each ATP, GTP, CTP, and UTP (in order to quantitate the yield of transcribed RNA) and were used as unlabeled transcripts for competition experiments. All transcripts were purified by electrophoresis through 5% polyacrylamide gels containing 7 M urea, eluted from the gel slices overnight at 4°C in 0.5 M ammonium acetate–1 mM EDTA, extracted once with phenol-chloroform, and precipitated with ethanol. ^{32}P-labeled transcripts were resuspended in water to 50,000 cpm/μl (about 3 ng of RNA per μl), and unlabeled transcripts were resuspended to about 300 ng of RNA per μl.

Preparation of cell extracts. Monolayers of secondary chicken embryo fibroblasts were grown in 150-mm diameter dishes to confluence. The cells were scraped off the plates and washed in cold phosphate-buffered saline (10). P100 salt wash (P100SW) extracts were prepared as described previously (16). Total protein concentration was determined by the Bio-Rad protein assay.

RNA-protein binding reaction and gel retardation analysis. The binding reaction was a modification of that described by Meerovitch et al. (22). Briefly, P100SW extract (750 ng except in titration experiments) was preincubated with 1 mg of poly(dI-dC) or alternatively poly(rI-rC) per ml, to suppress nonspecific binding, in 2.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.6)–12.5 mM KCl–1 mM $MgCl_2$–1.9% glycerol–0.01 mM dithiothreitol–0.75 mM ATP–1 mM GTP (binding buffer), in a final volume of 10 μl, at 30°C for 10 min. ^{32}P-labeled probes (5,000 cpm; about 0.3 ng of RNA except in titration experiments) were then added, and the mixture was incubated for 10 min at 30°C. In the case of competition experiments, various amounts of unlabeled transcripts were added to the reaction mixture either simultaneously with or after the ^{32}P-labeled probes, as indicated. The samples were then loaded onto 5% polyacrylamide gels containing 0.5× Tris-borate-EDTA and 5% glycerol and electrophoresed for 3 h at 30 mA at 20°C. The gels were dried and exposed to X-ray film with an intensifying screen at −80°C. Complex formation and free RNA were quantitated by scanning the dried gels with a Phosphorimager scanner (Molecular Dynamics).

UV-induced cross-linking and analysis of cross-linked proteins. P100SW extract (4 μg) was preincubated with 1 mg of poly(dI-dC) or poly(rI-rC) per ml in a final volume of 50 μl of binding buffer at 30°C for 10 min. A further incubation of 10 min at 30°C with the ^{32}P-labeled probes (125,000 cpm; about 7.5 ng of RNA) was followed by UV irradiation of the RNA-protein complexes for 20 to 60 min at 0°C with a short-wavelength lamp (Ultraviolet Products, San Gabriel, Calif.) held at 3 cm (20). In competition experiments, about 1.1 μg of unlabeled transcripts was added to the reaction mixtures either simultaneously with or after the ^{32}P-labeled probes (see text). The samples were then treated with 20 U of RNase T_1 and 20 μg of RNase A for 15 min at 37°C, heated at 70°C for 15 min in Laemmli sample buffer, and analyzed on 14% polyacrylamide–sodium dodecyl sulfate (SDS) gels (19).

RESULTS

Production of probes. To examine the ability of the Sindbis virus 5′ region to interact with cellular or viral proteins, we inserted a cDNA copy of the first 132 bases of the Sindbis virus genome into a transcription vector under the control of both SP6 and T7 RNA polymerase promoters, to give a plasmid referred to as pGEH (Fig. 1). Transcription of the plasmid by SP6 RNA polymerase gives rise to RNA transcripts of positive polarity, while those synthesized by T7 RNA polymerase are of negative polarity. The antisense

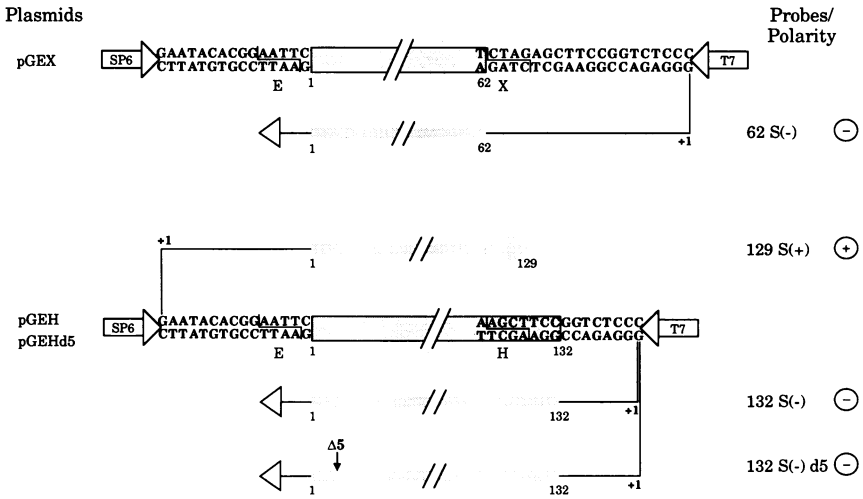

FIG. 1. Schematic diagram of in vitro transcripts used as probes in binding assays. All of the probes (^{32}P and ^{3}H labeled) were transcribed as described in Materials and Methods. +1 indicates transcription start. The shaded boxes represent Sindbis virus RNA sequence, with nucleotide numbers indicated under each box; SP6 and T7 promoters are presented as large boxed arrows. Restriction sites: E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. Δ5 indicates the position of deleted nucleotide 5.

transcript produced for use as a probe in these experiments was 145 bases in length and contained the complement of the first 132 bases of Sindbis virus RNA with 8 additional nucleotides from the vector pGemini at the 5′ end and 5 extra bases from the *Eco*RI linearizing site at the 3′ end; it is referred to as 132S(-) (Fig. 1). The plus-sense probe was 144 bases in length and contained the first 129 bases of Sindbis virus RNA with 15 extra bases from the vector pGemini at the 5′ end [129S(+)]. Antisense mutant probes were also produced by using clone constructs in which nucleotide A5 or G8 or both G8 and C36 (numbering in the genomic orientation) were deleted, to give probes referred to as 132S(-)d5, etc. [pGEHd5 and 132S(-)d5 are illustrated in Fig. 1]. We also produced a shorter minus-sense wild-type probe from a plasmid called pGEX that contained the first 62 bases of Sindbis virus RNA, 19 extra bases from the vector pGemini at the 5′ end, and 5 extra bases at the 3′ end (Fig. 1). Finally, a control probe (Bls) was produced that was a 127-residue SP6 transcript of pBEH that contained the sequence of restriction sites *Eco*RI and *Hin*dIII, the antisense sequence of the T7 RNA polymerase promoter, and pBluescript sequence up to the *Pvu*II restriction site.

Specific protein binding to the 3′ end of Sindbis virus minus-strand RNA. During development of this project, we tested several extract preparations, and the clearest results were obtained with a P100SW extract. When the radiolabeled minus-sense probe 132S(-) was incubated with P100SW extracts from uninfected secondary chicken embryo fibroblasts, we observed two retarded bands upon gel electrophoresis (Fig. 2, lane 2, bands I and II). Band I is specific to the antisense probe; when a non-Sindbis virus probe (Bls) or the plus-sense Sindbis virus probe 129S(+) was used, only a band migrating near the position of band II was present (Fig. 2, lanes 10 and 12).

Complex formation was found to be optimal in the presence of 0.25 to 0.65 M NaCl, 0.75 mM ATP, and 1 mM GTP (data not shown). If either the ATP or the GTP was omitted from the reaction, the amount of complex formed was reduced threefold.

To examine the specificity of the different complexes observed with the minus-sense probe, we performed competition experiments in which an excess of unlabeled competitor, either unlabeled 132S(-) or the non-Sindbis virus Bls probe, was added to displace the equilibrium. We found that only the Sindbis virus competitor interfered with the formation of band I (Fig. 2, lanes 3 to 5); this band was still present after the addition of 150 ng of non-Sindbis virus competitor (Fig. 2, lanes 6 to 8). We conclude that band I is formed upon specific binding of cellular proteins to the Sindbis virus minus-sense probe.

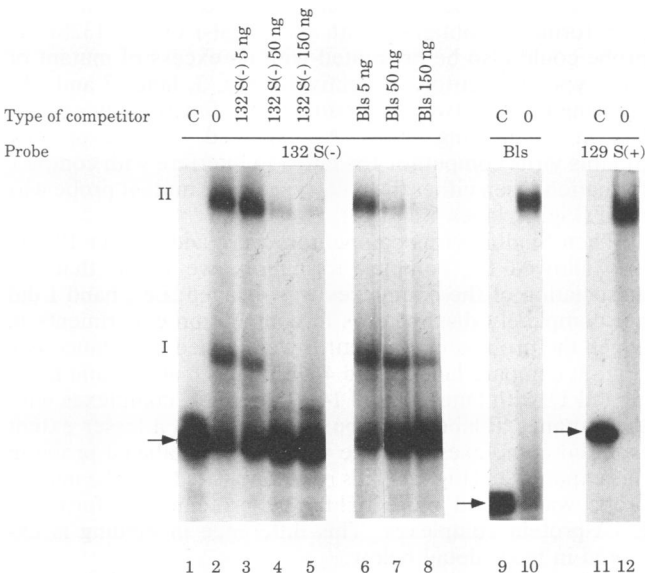

FIG. 2. Specificity of complex formation. Complex formation was performed as described in Materials and Methods, using the 132S(-) ^{32}P-labeled probe (lanes 1 to 8), Bls probe (lanes 9 and 10), or 129S(+) probe (lanes 11 and 12). The type and quantity of competitor are indicated above each lane. Lane C, no incubation with extract; lane O, binding assay in the absence of competitor. ^{32}P-labeled probe and ^{3}H-labeled competitor were added at the same time. I and II refer to two major retarded bands; band I is specific for the 132S(-) probe, whereas band II is not (see text). Arrows indicate free probe.

In contrast to the results with band I, band II formed with the minus-sense probe was abolished by both the Sindbis virus and non-Sindbis virus competitors, indicating that this band probably arose from a nonspecific interaction (Fig. 2, lanes 2 to 8). The intensity of band II was also found to vary, depending on the particular batch of extract used. In Fig. 2, this band was very intense, while it was hardly visible in similar assays shown below (see Fig. 3, 5, and 7), in which other batches of extracts were used. With a few extracts, a third band was also present (see Fig. 3); this band, called III, migrated between bands I and II and, when present, was a faint band formed only with the antisense probe; competition experiments indicated that it formed nonspecifically.

In early experiments, we also examined extracts from infected cells and found band shifts similar to those with uninfected cell extracts, although there were small differences in the amount of complex formed (not shown). We concluded that the complexes observed arose mainly from interactions with cellular proteins and subsequently used only uninfected extracts.

Protein binding with probes containing defined deletions. Having shown that band I contained a specific complex of cellular proteins with RNA sequences from the 3′ end of the complement of Sindbis virus RNA, we wanted to compare the binding of wild-type probes with that of probes transcribed from mutagenized derivatives. Mutant 5NTd(5) bears a lethal deletion of nucleotide A5 (25), and an antisense probe was made that was identical to the wild-type probe with the exception that nucleotide 5 (numbering in the genomic sense) was deleted [probe 132S(-)d5] (Fig. 1). In gel retardation assays, we observed a band comigrating with band I when the mutant probe was used (Fig. 3, lanes 2 and 9). Competition experiments showed that only the Sindbis virus competitors interfered with complex formation by either ^{32}P-labeled probe (Fig. 3, lanes 6 and 14). Furthermore, cross-competition experiments showed that the complex formation obtained with the 132S(-) or the 132S(-)d5 probe could also be prevented with an excess of mutant or wild-type competitor, respectively (Fig. 3, lanes 7 and 13), showing that the two competitors were functionally equivalent in the binding assay. As expected, the use of non-Sindbis virus competitor Bls failed to interfere with complex formation when either the wild-type or the mutant probe was used (Fig. 3, lanes 5 and 12).

When Sindbis virus competitors were added after 10 min was allowed for complex formation, we found that the dissociation of the complexes was delayed; i.e., band I did not completely disappear as in competition experiments in which the probe and competitor were added simultaneously (Fig. 3; compare lanes 3 and 4 with lanes 6 and 7, and lanes 10 and 11 with lanes 13 and 14). Moreover, complexes with the mutant ^{32}P-labeled probe dissociated to a lesser extent than did complexes with the wild-type ^{32}P-labeled probe in this experiment (Fig. 3). This result indicated that the mutant probe was bound more tightly by protein(s) to form the RNA-protein complexes. This difference in binding is explored in more detail below.

We also examined the importance of two other mutations on complex formation. Sindbis virus deletion mutants 5NTd(8) and 5NTd(8,36) are phenotypically thermosensitive (25), with altered RNA synthesis even at the permissive temperature (30). Probes 132S(-)d8 and 132S(-)d8,36, obtained from the corresponding deletion mutants, were used in complex formation and competition experiments. They gave results identical to those obtained with use of the wild-type 132S(-) probe, suggesting that nucleotides 8 and 36

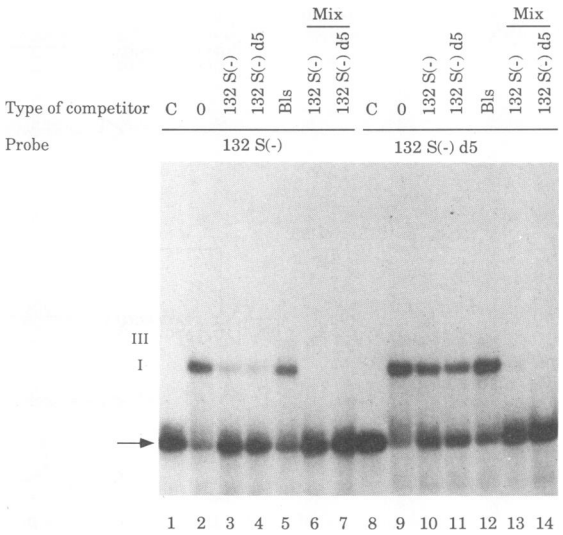

FIG. 3. Comparison of complex formation with use of either the 132S(-) or 132S(-)d5 ^{32}P-labeled probe. Complex formation was performed as described in Materials and Methods, using either the 132S(-) (lanes 1 to 7) or 132S(-)d5 (lanes 8 to 14) ^{32}P-labeled probe. The type of competitor (about 45 ng) is indicated above each lane. Lane C, no incubation with extract; lane O, binding assay in the absence of competitor. Mix indicates that the probe and competitor were added concomitantly; otherwise the ^{32}P-labeled probe was added first and the competitor was added after complex formation had occurred. I indicates the major retarded band I which is specifically formed with the Sindbis virus probes, and III refers to a minor band which appears to form nonspecifically (see text). The arrow indicates free probe.

are not directly involved in the RNA-protein interactions responsible for the complex formation being analyzed here.

Quantitative analyses of the RNA-protein interactions. To compare complex formation with the wild-type 132S(-) probe and the mutant 132S(-)d5 probe in greater detail, a constant amount of cellular extract was titrated with increasing concentrations of each probe and the extent of complex formation was analyzed by gel retardation. The proportion of the probes in the retarded bands was quantitated, and the amount of specific complex formed is plotted against the amount of total specific binding site available in Fig. 4A. The data were transformed (Fig. 4B) as described by Calzone et al. (6) to obtain an estimate of K_r, which is the ratio of the affinity constant for the specific binding reaction (K_s) to the affinity constant for the nonspecific binding reaction with the large excess of nonspecific RNA [poly(dI-dC)] present during the assays (K_n) ($K_r = K_s/K_n$). The data in Fig. 4B were fitted with least-squares lines, and K_rs were calculated from the slopes of these lines. The values of K_r for the wild-type and the mutant probes were very similar (wild-type $K_r = 3.2 \times 10^5$; mutant $K_r = 4.3 \times 10^5$), although we consistently found the wild-type K_r to be slightly smaller than the mutant K_r in several independent experiments.

In a second approach to determine the relative affinities of the binding proteins for the wild-type and mutant probes, we measured the half-life ($t_{1/2}$) of the complexes formed with either the 132S(-) or 132S(-)d5 probe. For this purpose, complex formation with the probe was allowed to occur, and then an excess of unlabeled competitor was added. After various periods of incubation in the presence of the competitor, the samples were analyzed on gels (Fig. 5A), and the

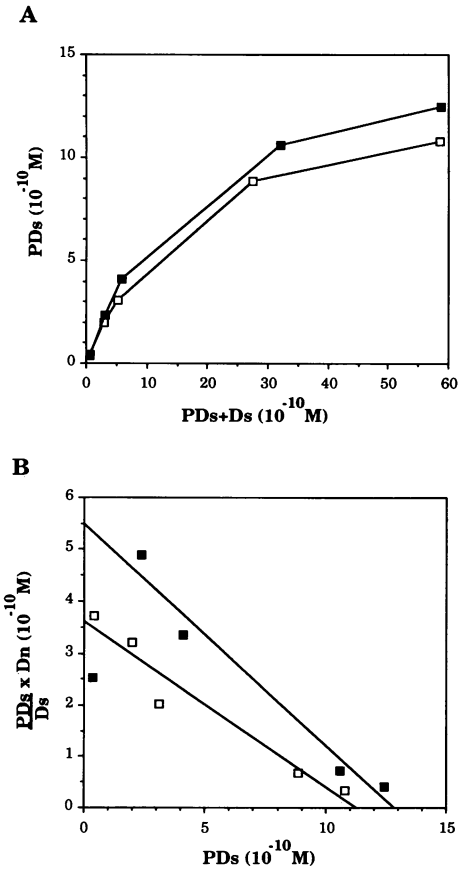

FIG. 4. Titration of complex formation. Binding reactions were performed as described in Materials and Methods, with addition of an increasing quantity of the 132S(-) (□) or 132S(-)d5 (■) ^{32}P-labeled probe. The amount of complex formed was quantitated by scanning the gel with a Phosphorimager scanner. (A) Plot of the concentration of specific complex formed (PDs) versus the concentration of specific complex (PDs) plus free RNA probe (Ds). (B) Transformation of the data by the method of Calzone et al. (6), where Dn is the concentration of nonspecific binding sites. The slope of the line in panel B is $-K_r$, and the intercept is P_0K_r, where P_0 is the concentration of binding protein in the assay (6).

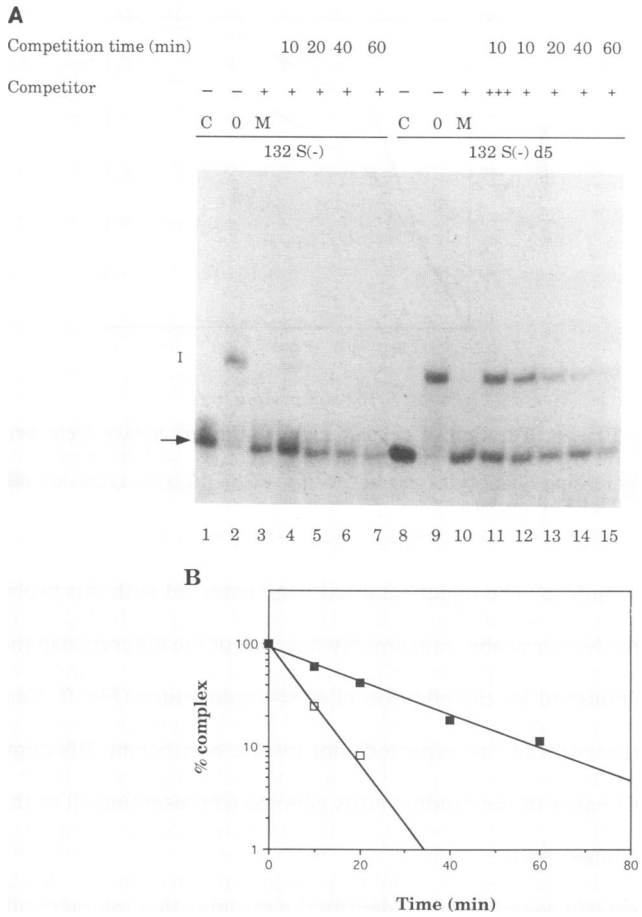

FIG. 5. Time course of complex dissociation. Binding experiments were performed as described in Materials and Methods, using either the 132S(-) (lanes 1 to 7) or 132S(-)d5 (lanes 8 to 15) ^{32}P-labeled probe. 132S(-) or 132S(-)d5 competitor was added either at the same time as the corresponding ^{32}P-labeled probe or after complex formation. (A) Gel retardation assay. + indicates that competitor (about 45 ng) was added. Lane C, no incubation with extract; lane O, binding assay in the absence of competitor; lane M, the probe and competitor were added concomitantly. After addition of unlabeled competitor, reaction mixtures were kept at 30°C for the time indicated above the lanes before being loaded onto a gel. +++ indicates addition of about 135 ng of 132S(-)d5 competitor. I refers to major retarded band I; the arrow indicates free probe. (B) Graphic representation of dissociation kinetics with use of the 132S(-) (□) or 132S(-)d5 (■) ^{32}P-labeled probe and their corresponding competitors. The data were obtained by quantitation of the results in panel A.

quantity of complexes remaining was determined as a function of time (Fig. 5B). With the wild-type probe, the $t_{1/2}$ was 5.5 min (apparent dissociation constant $[k_d] = 2.1 \times 10^{-3}$ s^{-1}), whereas with the mutant probe it was 16 min ($k_d = 0.72 \times 10^{-3}$ s^{-1}). As a control, we showed that adding the probe and its unlabeled competitor simultaneously resulted in complete displacement of the equilibrium in both cases (Fig. 5A, lanes 3 and 10). Thus, the wild-type complex dissociates threefold faster than the mutant complex, and the mutant probe is bound more tightly by the proteins involved in the Sindbis virus-specific complexes.

The observed $t_{1/2}$ for the mutant probe was not changed when the competitor concentration was increased by threefold (Fig. 5, lane 11). Quantitation of band I in cross-competition experiments (Fig. 3, lanes 3, 4, 10, and 11) did not show any significant difference in the half-lives determined. However, for concentrations of competitor under 40 ng, the mutant competitor was slightly more effective (about 15%; not shown).

The reciprocal experiment to the probe titration was performed by using a constant amount of probe to titrate an increasing quantity of extract (Fig. 6). We found that the quantity of extract needed to form 50% of the final amount of complex that could be formed with either the mutant or wild-type probe was about 250 ng. For quantities of extract above 150 ng, the mutant probe was able to form 1.5 times more complexes than the wild-type probe, again demonstrating that the mutant probe has a greater affinity for the proteins involved in complex formation.

RNA-protein interactions with a shorter probe. To extend our knowledge of the sequence requirements for protein interactions, we shortened the wild-type probe to include the complement of only the first 62 bases of the Sindbis virus

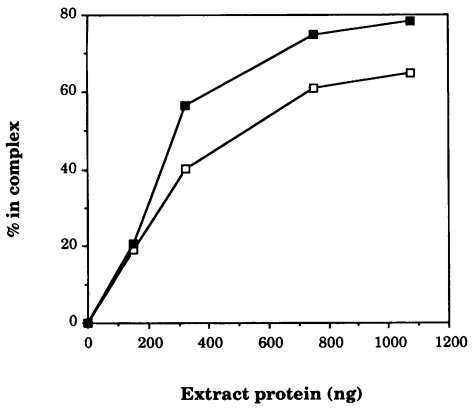

FIG. 6. Titration of cellular extract. Binding assays were performed as described in Materials and Methods, with addition of an increasing quantity of extract to the 132S(-) (□) or 132S(-)d5 (■) ^{32}P-labeled probe.

sequence. The major retarded band detected with this probe migrated slightly faster than the band I complex formed with the longer probe, presumably because of the difference in the sizes of the probes (Fig. 7, denoted I*). This band was abolished by the addition of 62S(-) competitor (Fig. 7, lane 4), as well as by 132S(-) and 132S(-)d5 competitors (not shown), but, as expected, not by the nonspecific Bls competitor (not shown). This result suggested that only the first 62 bases of the Sindbis virus genome (representing all of the 5′ NTR) were sufficient for the formation of the complexes studied here.

Identification of cellular proteins involved in the RNA-protein complexes. To identify the proteins that interact with

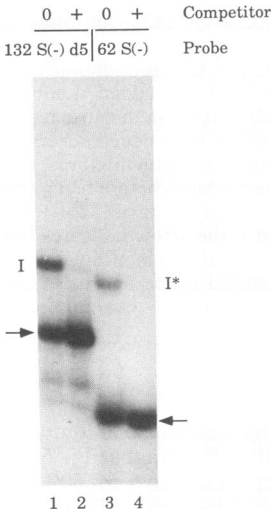

FIG. 7. Comparison of complex formation with use of the 132S (-)d5 and 62S(-) probes. Binding experiments were performed as described in Materials and Methods, using either the 132S(-)d5 (lanes 1 and 2) or 62S(-) (lanes 3 and 4) ^{32}P-labeled probe in the absence of competitor (lanes 1 and 3) or in the presence of about 45 ng of the 132S(-)d5 competitor (lane 2) or 62S(-) competitor (lane 4). I refers to the position of major retarded band I observed with use of the 132S(-)d5 ^{32}P-labeled probe; I* refers to major retarded band I observed with use of the 62S(-) ^{32}P-labeled probe; arrows indicate free probe.

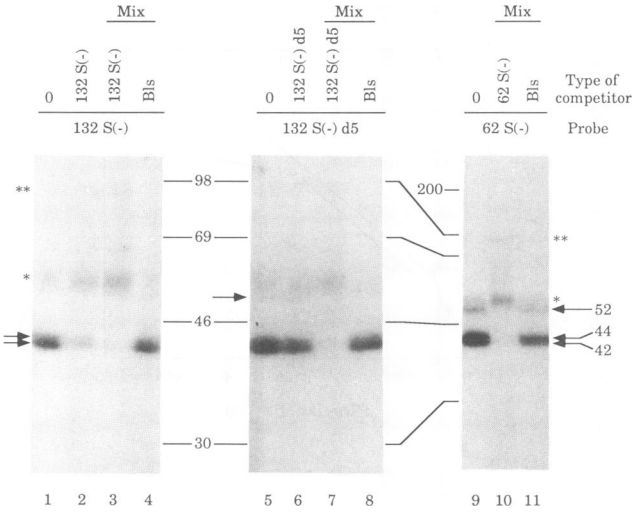

FIG. 8. Analysis of proteins UV cross-linked to ^{32}P-labeled probes. Binding assays and UV cross-linking were performed as described in Materials and Methods, using the 132S(-) (lanes 1 to 4), 132S(-)d5 (lanes 5 to 8), or 62S(-) (lanes 9 to 11) ^{32}P-labeled probe. Samples were analyzed on an SDS–14% polyacrylamide gel. The type of competitor (about 45 ng) is indicated above each lane. Lane O, binding assay in absence of competitor. Mix indicates that the probe and competitor were added concomitantly. In lanes 2 and 6, the indicated competitor was added after complex formation. 42, 44, and 52 refer to three cellular proteins specifically labeled by the Sindbis virus RNA probes with apparent molecular sizes of 42, 44, and 52 kDa, respectively; * and ** indicate the positions of two proteins of 56 and 95 kDa, respectively, that bind the Sindbis virus probes nonspecifically.

the Sindbis virus antisense RNA probes, we irradiated the complexes with UV light to cross-link the probes to the proteins that were bound to them. The majority of the probe was then removed with RNase, and the proteins that were labeled by transfer of ^{32}P from the probe were analyzed on SDS-containing polyacrylamide gels. With the wild-type 132S(-) probe, the wild-type 62S(-) probe, and the 132S(-)d5 mutant probe, at least five bands with apparent sizes of 42, 44, 52, 56, and 95 kDa were seen on the autoradiogram (Fig. 8). The 42- and 44-kDa proteins, and to a lesser extent the 52-kDa protein, appeared to form specific complexes with the Sindbis virus probes. First, these proteins were not labeled when the Bls probe was used to form the complexes (not shown). Second, labeling of the 42- and 44-kDa proteins, which clearly interacted with both the long and short Sindbis virus probes, could be abolished with Sindbis virus competitors (Fig. 8, lanes 3, 7, and 10) but not with the Bls competitor (Fig. 8, lanes 4, 8, and 11). The 52-kDa protein interacted with the Sindbis virus 62-nucleotide probe; it disappeared upon competition with Sindbis virus competitor but was only slightly affected by the nonspecific competitor (Fig. 8, lanes 10 and 11, respectively). This protein also appeared to be labeled with the long probes, both the wild-type and d5 mutant probes, but the labeling was weak and the band was too diffuse to be accurately assayed. In fact, all of the protein bands in such experiments were diffuse in comparison with molecular weight marker bands, possibly because of the UV light treatment or removal of the RNA probe to various extents by RNase treatment. Thus, the situation with the 52-kDa protein in the assays with the long probes is not clear.

The 56- and 95-kDa proteins appeared to interact with the probes nonspecifically. Curiously, labeling of the 56-kDa protein was not abolished by adding unlabeled 62S(−) probe as a competitor; instead, the amount of labeled protein appeared to increase (Fig. 8, lanes 9 and 10). Similarly, nonspecific labeling of the 56-kDa protein by the 132S(−) ^{32}P-labeled probe was slightly increased by the addition of unlabeled 132S(−) probe (Fig. 8, lanes 1 and 3 and lanes 5 and 7). Such an increase in nonspecific transfer of label to a protein upon addition of unlabeled probe has been observed in UV-cross-linking studies of p100, which binds nonspecifically to nucleotides 559 to 624 of the poliovirus RNA noncoding region (22).

It is not clear whether the 42- and 44-kD bands involved in complex formation with the Sindbis virus RNA probes arise from two different proteins that differ slightly in size, or whether they result from one protein that migrates as a doublet because of modification(s). Such migration as a double band by a binding protein has been described for the ABF1 factor, a 135-kDa protein that binds to a sequence called the polymerase upstream box sequence in yeast cells (8), as well as for the cellular p57 RNA-binding protein involved in the cap-independent translation of encephalomyocarditis virus RNA (17).

DISCUSSION

Binding of cell proteins to the 3′ end of the Sindbis virus minus strand. We have used short riboprobes to assay for the interaction of cellular proteins with the 3′ end of Sindbis virus minus-strand RNA. It is usually assumed that the affinity constant in this type of nucleic acid-protein interaction is determined by the dissociation constant, k_d, as the association constant, k_a, is normally a function simply of diffusion and thus does not vary from probe to probe. A threefold difference in the k_d for the wild-type and mutant probes would then predict a threefold difference in the affinity constant K_s ($= k_a/k_d$), and therefore in K_r ($=K_s/K_n$), for these two probes. We found only a slight difference in the K_rs, about 1.4-fold, suggesting that there may be a difference in the mutant and wild type k_a, possibly caused by changes in RNA conformation. It seems more likely, however, that the K_r estimates are somewhat inaccurate because of the difficulties of quantitating low concentrations of complex necessary for determination of the slope, and that the affinity constants probably do differ by a factor of 3. In any event, the mutant k_d and the wild-type k_d are clearly different, and this difference could account for the mutant phenotype.

It is noteworthy that the values of K_r and of k_d are similar to values found for the interaction of proteins with DNA sequence elements involved in transcriptional control in the developing sea urchin. Calzone et al. (6) obtained values for K_r from 1×10^5 to 19×10^5 for 23 different protein binding sites, compared with our estimates of 3.2×10^5 and 4.3×10^5 for the wild-type and mutant Sindbis virus sites. They also measured k_d for two sea urchin sites, using an assay similar to the one used here, and obtained values of 8.0×10^{-4} s^{-1} for one binding site and 2.4×10^{-4} s^{-1} for a second site, comparable to our estimates of 21×10^{-4} and 7×10^{-4} s^{-1} for the wild-type and mutant Sindbis virus probes. Finally, they obtained an estimate for K_n of 550. This value for K_n would predict an affinity constant K_s for the interactions with the Sindbis virus probes of about 2×10^8. It is thus clear that the binding interactions studied here are of a suitable magnitude to be important in the development of the virus life cycle.

Binding of proteins to structural elements in RNA virus genomes. Nontranslated regions of RNA virus genomes have been found to be involved in binding of proteins required for translation of the RNA or for its replication. In a number of cases, stem-loop structures have been shown to act as protein binding sites. Thus, the 5′ NTR of picornavirus RNAs can be folded into a series of stem-loop structures. The conservation of a number of these structures among picornaviruses suggests that they can play an important role in virus replication (34), and a number of studies have shown that these structures are important for replication or translation of the viral RNA (17, 31, 36). The 5′ NTR of poliovirus RNA was shown to bind a cellular protein p52 thought to be involved in initiation of translation of poliovirus RNA (7, 22), while a 57-kDa cellular protein was shown to bind specifically to the 5′ end of encephalomyocarditis virus RNA and to be involved in cap-independent translation (17). In the latter case, the binding of this protein was shown to require stem-loop structures in the 5′ NTR. The 5′ NTR must also be involved in RNA synthesis; in the case of poliovirus, it has been shown that $3C^{pro}$ and $3D^{pol}$ bind to a stem-loop structure in the 5′ NTR and promote plus-strand RNA replication (1, 2).

The 3′ NTR of rubella virus RNA is capable of binding three cellular proteins that may be involved in the replication of the virus (24). A stem-loop structure appears to be important for the binding. Although deletion of a G residue and a C residue hypothetically base paired in the stem of a stem-loop structure at the 3′ end of the genomic RNA, or of the G residue alone, had no effect on complex formation, deletion of two unpaired uridine residues in the stem-loop structure led to a drastic reduction in complex formation (24).

It is also known that binding of the coat protein of bacteriophage R17 to a hairpin loop structure in the initiation region of the replicase gene serves a regulatory role in the phage life cycle. Deletion of a bulged adenosine residue led to a dramatic decrease in the specific binding of the coat protein, demonstrating the importance of the hairpin-loop structure for binding (37).

Binding of cellular proteins may be required for togavirus RNA replication. The mutagenesis data of Niesters and Strauss (25) suggested that the 5′-terminal hairpin structure, or the 3′-terminal complement in the minus strand, served as a binding site for cellular proteins. The results presented here on the effects of binding of cellular proteins to the 3′ end of the minus strand of wild-type and mutant d5 RNAs are consistent with the hypothesis that the 3′-terminal structure in the minus strand is bound by cellular proteins to promote RNA replication, and we propose that the binding of cellular proteins observed in our experiments represents one of several crucial steps in the initiation of plus-strand RNA synthesis from a minus-strand template. It is interesting to note that in other cases described above, a severe reduction of the binding activity was observed when mutations were introduced into structures bound by proteins, while a deletion of a comparable unpaired nucleotide in the Sindbis virus structure led to a threefold increase in the half-life of the complex. It is unclear whether the effect of the d5 mutation arises because this nucleotide is a contact residue for binding, or whether the deletion leads to an altered secondary structure which functions improperly. Biebricher et al. (4) showed that different folding of a region of Qβ bacteriophage RNA may destroy its ability to function as a template for replication. Assuming that our model is correct, it is also unclear whether the slower dissociation rate of cellular

protein(s) from Sindbis virus d5 initiation complexes during the initial stages of viral RNA replication leads to a reduction or suppression of further replicase activity, or whether the abolition of a binding site for another protein involved in complex formation is responsible for the lethality of the d5 mutation. In any event, in mutants 5NTd(8) and 5NTd(8,36), the required structure for RNA replication might be abolished only at the nonpermissive temperature, although a destabilization of the structure at the permissive temperature might be responsible for the altered RNA synthesis under these conditions. In our binding assay experiments, these two probes were indistinguishable from the wild-type probe.

Mutagenesis experiments of Kuhn et al. (18) led to the hypothesis that cellular factors, presumably different from those studied here, also bind to the 3′ NTR of Sindbis virus to promote minus-strand synthesis from a plus-strand template. As described above, Nakhasi et al. (24) identified cellular factors that bind to the 3′ NTR of rubella virus, a virus related to Sindbis virus. Both Sindbis virus (genus *Alphavirus*) and rubella virus (genus *Rubivirus*) are classified as members of the family *Togaviridae*, and they appear to be related and descended from a common ancestor. Although only limited sequence identities are present throughout the genomes, the genome organizations of these two virus groups, as well as many aspects of their replication and structure, are very similar (9). It seems likely that these viruses also share the property of using cellular proteins to promote both plus-strand and minus-strand synthesis. In Sindbis virus, a 51-nucleotide element capable of forming two stem-loop structures, present in the coding region of nsP1, has also been hypothesized from mutational studies to bind cellular proteins (26). This element is not highly conserved in rubella virus (9), and it is unclear whether it serves the same function in this virus.

The identities of the cellular proteins that bind to Sindbis virus RNA are clearly of interest. Our cross-linking experiments identified two cellular proteins that were specifically involved in complex formation with the 3′ end of the minus strand, as well as a third protein that may also react specifically with the viral sequences. These proteins may be ribosome associated, since they are present in the salt wash supernatant of cellular extract pellets, which is a common way of isolating ribosome-associated factors. There is perhaps analogy to the situation with bacteriophage Qβ replicase in which three cell proteins normally associated with protein synthesis form components of the replicase (5). We are currently attempting to isolate sufficient quantities of these proteins to allow their characterization.

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REFERENCES

1. **Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5′ end of poliovirus RNA. Cell **63**:369–380.
2. **Andino, R., G. E. Rieckhof, D. Trono, and D. Baltimore.** 1990. Substitutions in the protease ($3C^{pro}$) gene of poliovirus can suppress a mutation in the 5′ noncoding region. J. Virol. **64**:607–612.
3. **Barton, D. J., S. G. Sawicki, and D. L. Sawicki.** 1988. Demonstration in vitro of temperature-sensitive elongation of RNA in Sindbis virus mutant *ts*6. J. Virol. **62**:3597–3602.
4. **Biebricher, C. K., S. Diekman, and R. Luce.** 1982. Structural analysis of self-replicating RNA synthesis by Qβ replicase. J. Mol. Biol. **154**:629–648.
5. **Biebricher, C. K., and M. Eigen.** 1988. Kinetics of RNA replication by Qβ replicase, p. 1–18. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics. CRC Press, Boca Raton, Fla.
6. **Calzone, F. J., N. Theze, P. Thiebaud, R. L. Hill, R. J. Britten, and E. H. Davidson.** 1988. Developmental appearance of factors that bind specifically to *cis*-regulatory sequences of a gene expressed in the sea urchin embryo. Genes Dev. **2**:1074–1088.
7. **Del Angel, R. M., A. G. Papavassilliou, C. Fernandez-Tomas, S. J. Silverstein, and V. R. Racaniello.** 1989. Cell proteins bind to multiple sites within the 5′ untranslated region of poliovirus RNA. Proc. Natl. Acad. Sci. USA **86**:8299–8303.
8. **Della Seta, F., I. Treich, J. M. Buhler, and A. Sentenac.** 1990. ABF1 binding sites in yeast RNA polymerase genes. J. Biol. Chem. **265**:15168–15175.
9. **Dominguez, G., C.-Y. Wang, and T. K. Frey.** 1990. Sequence of the genome RNA of rubella virus: evidence for genetic rearrangement during togavirus evolution. Virology **177**:225–238.
10. **Dulbecco, R., and M. Vogt.** 1954. One-step growth curves of western encephalomyelitis virus grown in vitro and analysis of the virus yields from single cells. J. Exp. Med. **99**:183–199.
11. **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1988. A novel superfamily of nucleoside triphosphate-binding motif containing proteins which are probably involved in duplex unwinding in DNA and RNA replication and recombination. FEBS Lett. **235**:16–24.
12. **Hahn, Y. S., A. Grakoui, C. M. Rice, E. G. Strauss, and J. H. Strauss.** 1989. Mapping of RNA^- temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. J. Virol. **63**:1194–1202.
13. **Hahn, Y. S., E. G. Strauss, and J. H. Strauss.** 1989. Mapping of RNA^- temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J. Virol. **63**:3142–3150.
14. **Hardy, W. R., and J. H. Strauss.** 1989. Processing the nonstructural proteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in *cis* and *trans*. J. Virol. **63**:4653–4664.
15. **Hayes, R. J., and K. W. Buck.** 1990. Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase. Cell **63**:363–368.
16. **Huismans, H., A. A. van Dijk, and A. R. Bauskin.** 1987. In vitro phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. J. Virol. **61**:3589–3595.
17. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57 kDa RNA-binding protein. Genes Dev. **4**:1560–1572.
18. **Kuhn, R. J., Z. Hong, and J. H. Strauss.** 1990. Mutagenesis of the 3′ nontranslated region of Sindbis virus RNA. J. Virol. **64**:1465–1476.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Leibold, E. A., and H. N. Munro.** 1988. Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs. Proc. Natl. Acad. Sci. USA **85**:2171–2175.
21. **Levis, R., S. Schlesinger, and H. V. Huang.** 1990. The promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. J. Virol. **64**:1726–1733.
22. **Meerovitch, K., J. Pelletier, and N. Sonenberg.** 1989. A cellular protein that binds to the 5′ non coding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3**:1026–1034.
23. **Mi, S., R. Durbin, H. V. Huang, C. M. Rice, and V. Stollar.**

1989. Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. Virology **170**:385–391.

24. **Nakhasi, H. L., T. A. Rouault, D. J. Haile, T.-Y. Liu, and R. D. Klausner.** 1990. Specific high-affinity binding of host cell proteins to the 3′ region of rubella virus RNA. New Biol. **2**:255–264.
25. **Niesters, H. G. M., and J. H. Strauss.** 1990. Defined mutations in the 5′ nontranslated sequence of Sindbis virus RNA. J. Virol. **64**:4162–4168.
26. **Niesters, H. G. M., and J. H. Strauss.** 1990. Mutagenesis of the conserved 51 nucleotide region of Sindbis virus. J. Virol. **64**:1639–1647.
27. **Ou, J.-H., C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss.** 1982. Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. Proc. Natl. Acad. Sci. USA **79**:5235–5239.
28. **Ou, J.-H., E. G. Strauss, and J. H. Strauss.** 1983. The 5′-terminal sequences of the genomic RNAs of several alphaviruses. J. Mol. Biol. **168**:1–15.
29. **Ou, J.-H., D. W. Trent, and J. H. Strauss.** 1982. The 3′-noncoding regions of alphavirus RNAs contain repeating sequences. J. Mol. Biol. **156**:719–730.
30. **Pardigon, N.** Unpublished data.
31. **Racaniello, V. R., and C. Meriam.** 1986. Poliovirus temperature-sensitive mutant containing a single nucleotide deletion in the 5′ noncoding region of the viral RNA. Virology **155**:498–507.
32. **Raju, R., and H. V. Huang.** 1991. Analysis of Sindbis virus promoter recognition in vivo, using novel vectors with two subgenomic mRNA promoters. J. Virol. **65**:2501–2510.
33. **Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang.** 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature sensitive marker, and in vitro mutagenesis to generate defined mutants. J. Virol. **61**:3809–3819.
34. **Rivera, V. M., J. D. Welsh, and J. V. Maizel.** 1988. Comparative sequence analysis of the 5′ noncoding region of the enteroviruses and rhinoviruses. Virology **165**:42–50.
35. **Strauss, E. G., and J. H. Strauss.** 1986. Structure and replication of the alphavirus genome, p. 35–90. *In* S. Schlesinger and M. J. Schlesinger (ed.), The Togaviridae and Flaviviridae. Plenum Publishing Corp., New York.
36. **Trono, D., R. Andino, and D. Baltimore.** 1988. An RNA sequence of hundreds of nucleotides at the 5′ end of poliovirus RNA is involved in allowing virus protein synthesis. J. Virol. **62**:2291–2299.
37. **Wu, H.-N., and O. C. Ulhenbeck.** 1987. Role of a bulged A residue in a specific RNA-protein interaction. Biochemistry **26**:8221–8227.